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Synthesis of a Chemical Probe for RNA Aptamer

by

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Introduction

Our current understanding of DNA's role in providing instructions for the genetic code have led to an abundance of applications in daily life including forensic testing, genetic screening, and research. Over the past two centuries, deoxyribose nucleic acid (DNA) has transitioned from an obscure molecule with unknown structure and function to the very foundation of modern biology. From Gregor Mendel's pea plants to the Griffith experiment to Avery, McCarty, and Macleod to Hershey and Chase to Watson and Crick and all of the scientists in between, the story of DNA has provided researchers with the tools to understand organisms and many types of viruses.

DNA and ribonucleic acid (RNA) make up the two major classes of nucleic acids: both are polymers that are composed of monomeric nucleotide units. Each nucleotide consists of three molecular fragments; deoxyribose or ribose sugar molecules in DNA and RNA respectively, a phosphate group, and a nitrogenous base (***Figure 1***). The four nitrogenous bases in nucleic acids are adenine (A), cytosine (C), guanine (G), and thymine (T). In RNA, uracil (U) takes place of thymine (T). Nitrogenous bases are bound to a sugar molecule via a B-glycosyl linkage. Consecutive linkages make up the sugar phosphate backbone in nucleic acids. Each monomeric unit in the backbone consists of a 5' attachment of phosphate groups on a sugar molecule and its corresponding 3' ester linkage to the carbon of an adjacent nucleotide. In DNA, two sugar phosphate backbones run antiparallel to one another and twist to form a double

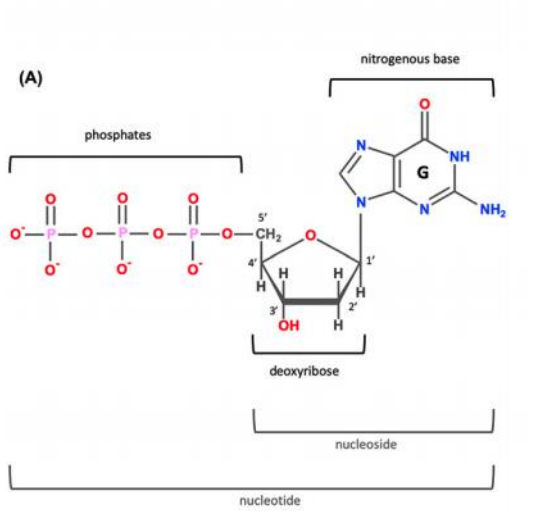


***Figure 1**: Structure of DNA (Minchin et al., 2019)*

helix structure bound by hydrogen bonds between a purine and a pyrimidine[1]. Specific base pairing between guanine (G) and cytosine (C) as well as adenine (A) and thymine (T)/ uracil (U) is essential for life and the structure of nucleic acids.

Found inside the nucleus of eukaryotic cells in the form of chromatin or heterochromatin, DNA acts to provide a long-term storage facility of genetic information and is able to copy itself via replication. Other DNA molecules found within the eukaryotic genome may also serve other structural functions, regulate gene expression, and provide the genetic information necessary for the transcription of messenger RNA (mRNA)[2] . Transcription begins when RNA polymerase binds to a promoter region of DNA: this allows for elongation of the mRNA strand (***Figure 2***). When the RNA polymerase recognizes and transcribes the terminator sequence on DNA, the mRNA molecule is transported to the cytoplasm by ribosomal RNA (rRNA). Inside the cytoplasm, ribosomes bind to the newly synthesized mRNA strand and recruit transfer RNA (tRNA) to bind at a site- specific codon region. The amino acids bound to each molecule of tRNA make up a finalized protein which may undergo further modifications in a neighboring cell organelle. This process is often referred to as the central dogma of life.

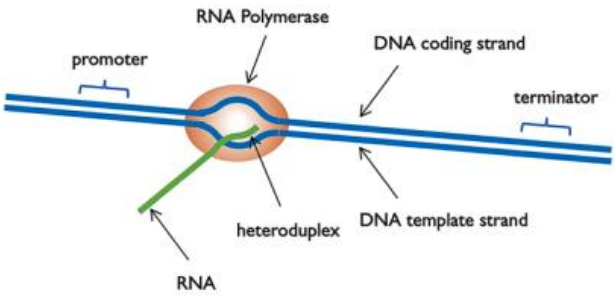


Figure 2: *Transcription (Minchin et al., 2019)*

Unlike DNA, RNA molecules are easier to break down and do not have a stable secondary structure[2]. The 2' hydroxyl group on ribose sugar results different behavior in chemical environments compared to the 2' hydrogen in deoxyribose sugar. However, the single stranded nature of RNA allows for a more dynamic range of possible 3-D structures such as hairpin loops,

multiloops, or internal loops. Due to the diversity in shape of RNA, it's function serves many essential purposes including ligand binding, catalysis of chemical reactions, protein synthesis and recognition, splicing, and other chemical modifications[3]. The activity of RNA is extremely dependent on its surrounding chemical environment- understanding this structure and its vital role played in the natural world is desired by researchers.

One way that RNA directly interacts with proteins involves the formation of ribonucleoprotein complexes (RNP) which can contain one or more protein subunits bound to one or more RNA molecules[3]. Research of RNA interactions is important as it provides information on how conformational changes initiate many essential molecular events. One example of how researchers are currently studying RNPs can be seen in *Structural Changes of RNA in Complex with Proteins in the SRP* where the relationship between RNA and protein binding was investigated. Through NMR, cryo-electron microscopy, and X-ray crystallography, researchers learned that RNA and protein binding can be classified as specific, protein specific, or non- specific[3].

RNA is also being used in gene therapy research. In a 2019 study published in Nature, Zhong and colleagues engineered a class of ribozymes that function to produce RNA switches that can cleave mRNA in a tightly regulated environment[4]. Without the addition of various protein complexes, reactions between RNA switches and mRNA could be manipulated and monitored more efficiently to monitor protein expression from an mRNA starting point. The removal of confounding variables in this study allowed for a more comprehensive understanding of RNA interactions in conjunction with gene-therapy techniques. It was reported that RNA switches were much safer than other gene-therapy techniques offered in healthcare.

Nucleic acids may also make up some of the genetic material in viruses. For example, COVID-19 contains genetic material in the form of RNA. RNA viruses can be classified according to sense or polarity of their RNA into negative-sense and positive-sense. Positive- sense RNA viruses have their genetic information in the form of mRNA. Upon infection by this type of virus, the host can immediately translate the viral material into viral proteins. Negative-sense RNA contains genetic information complimentary to mRNA and must be converted into positive-sense by an RNA polymerase. The coronavirus (CoV) family is characterized by an enveloped positive-sense single-stranded RNA virus. Coronavirus can specifically bind to entry receptors in a host through their spike proteins. Expression of proteins encoded for by the virus results in a respiratory immune response.

Nucleic acids are generally thought of as the blueprint to all life because they contain complex instructions for how organisms develop and replicate themselves. Nucleic acids can fold into complex structures depending on their chemical environment and adjust their function in the cell accordingly. However, the synthetic production of nucleic acids is a fairly new concept emerging from the explosion of technology in the 20th and 21st century. The generation of oligonucleotides with relatively non-biological functions began generation of nucleic acids invitro and amplification for specific use. Aptamers are nucleic acid molecules that can be extracted naturally from the environment or synthetically made: they can bind to a wide range of potential targets. In the following section we will examine the use of aptamers in nature and in research and identify the strengths and weaknesses of this class of biosensor.

Biology Background

Aptamers are short single-stranded oligonucleotide molecules that can bind at site-specific sequences of a desired target. In the late 20th century, Craig Turek and Larry Gold published a paper describing a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX), an aptamer selection approach[5]. Depending on the sequence of the aptamer, the ligand could be small molecules including peptides, cells, tissues, and proteins. Aptamer recognition of small molecule ligands results in a structural change at the tertiary structure level, an ideal characteristic of nucleic acid aptamers as molecule shape defines molecule function. SELEX, involves a library of single-stranded (ssDNA) molecules that produce ssDNA molecules or ssRNA molecules that can specifically bind to ligands (***Figure 3***). The original ssDNA library is composed of random sequences of oligonucleotides marked with primers on both the 3' and 5' end. After exposure to the target ligand, sequences that do not bind are removed from the library. On the other hand, sequences that do bind the molecule of interest are amplified by PCR to create double stranded DNA templates for use in-vitro synthesis of a desired DNA or RNA that serve as aptamers. The amplification process is repeated until the aptamer population is prepared for species with a target function[6].

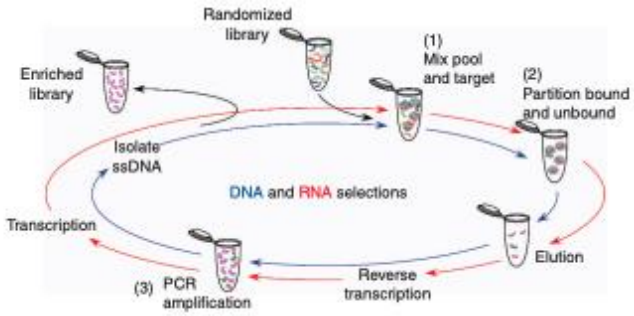


***Figure 3**: Selection of Aptamers (Maier et al., 2016)*

Aptamers are often compared to antibodies in regards to their biosensing abilities, but aptamers have been shown to display the greatest binding affinity, specifically in the pico-molar range, to their target molecules and are extremely stable[7]. To elaborate, aptamers can be

developed for almost any analyte and are smaller in size compared to antibodies, a preferrable characteristic when working in-vitro. For example, Chen and colleagues established a class of fluorescence-based aptamer biosensors that present with high detection of a target molecule HBC[8]. The fluorescence of HBC was enhanced 3000 fold when bound to aptamer D11 with reduced Mg^{2+} sensitivity and multiple rounds of optimization. This type of biosensor offered high sensitivity to HBC and real-time detection of the target. One disadvantage of aptamers is that SELEX takes several months to complete and can be very expensive compared to the simple synthesis of antibodies for a target of interest. However, SELEX and counter- SELEX yields aptamers that are extremely precise and do not bind to derivatives of their target molecule.

In consideration of another comparison category, aptamers can be stored for long periods of time as they are able to regain initial target binding sites after denaturation. In strike contrast to aptamers, antibody fragments cannot conserve their binding abilities once they have been denatured or compromised by things like enzyme activity, heat, or cleavage. Antibody fragments cannot be stored for a long period of time and have slight rejuvenation abilities[7]. Aptamers can be developed in non-conventional environments, allowing for more flexibility in procedure compared to their competing biosensor that requires compatible denaturing solvents and animals with an immune response[7]. The production of antibodies involves immunization of animals and blind- reliance on their immune systems depending on whether or not that animal can illicit an immune response in conjunction with researcher expectations. Several factors can impact this process including lack of antibody produced once extracted from the animal, complex immunogen preparations, and careful delivery of the antigen itself. Relying on the immune system to produce an antibody of interest for use in biosensing not as reliable as the SELEX

approach for aptamers and introduces more confounding variables[7]. Because aptamers have a larger range of target potential compared to antibodies, this class of biosensor play an advantageous role in clinical diagnostics and as therapeutic agents[9].

Aptamers exist naturally as ligand-binding elements of riboswitches, a regulatory element in mRNA molecules[10]. RNA molecules can act as receptors for other small molecules and modulate stages of gene expression. These small molecule metabolites can bind to regions of RNA, called riboswitches that affect transcription, splicing, or translation[11]. It is known that thiamine pyrophosphate, flavin mononucleotide, cobalamin, SAM, purines, glucosamine 6-phosphate, lysine, and glycine bind to riboswitches and every vitamin cofactor may bind to an RNA riboswitch. For example, the *thiM* riboswitch controls transcription of RNA. This small molecule can stabilize and mask or unmask functional RNA sequences: as ligand binding occurs on the *thiM* riboswitch, masking of a transcriptional anti-terminator region may take place to reveal transcriptional terminators[11]. Along with that, binding of a ligand to this riboswitch may hide ribosomal binding sites in order to prevent mRNA molecules from binding or associating with the ribosome. Some plants and fungi utilize *thiM* alternate splicing mechanisms of mRNA to consequentially affect protein expression. In analysis of the binding pattern of thiM riboswitch with thiamine pyrophosphate, pi stacking of thiamine and coordination around a Mg2+ ion takes place. To illustrate another example, the *GlmS* riboswitch binds to glucosamine-6-phosphate (G6P), acting as a cofactor for self-cleavage. The amine group of G6P acts as a Bronsted Acid allowing the *GlmS* riboswitch to protonate a leaving group during hydrolysis. Purines can also activate riboswitches within mRNAs that code for enzymes used in purine biosynthesis and transport. When more than one riboswitch is available in a nucleic acid transcript, they are all

able to sophisticatedly work together to control gene expression. All in all, riboswitches provide feedback control to biosynthetic machineries that synthesize the small metabolites they bind.

Aptamers selected from a random library of single-stranded DNA are found to imitate their natural equivalent, the riboswitches in both binding affinity and specificity. Emerging as potential targets for drug development and ensuring food safety and environment monitoring, aptamers are promising chemical elements in research. For example, the synthesis of Pegaptanib, a drug used in treatment of macular degeneration was the first aptamer approved for therapeutic use in humans[12]. Researchers began by first creating a DNA oligonucleotide library with a variable region followed by an T7- RNA polymerase promoter. The desired target, vascular endothelial growth factor (VEGF), was selected for based on its role as a regulator in pathological angiogenesis and inflammatory conditions like arthritis[12]. VEGF molecule is primarily responsible for restoring blood supply to tissues when deprived of oxygen or proper blood circulation. However, overexpression of VEGF is harmful in macular degeneration (AMD) causing an abnormal amount of blood vessels under the retina. In the attempt to design a drug that could treat this disease, the SELEX approach for aptamer selection and optimalization with 2'-F substitutions yielded the aptamer Pegaptanib[12]. Aptamer and target mixtures invitro were performed to trigger the growth of blood vessels. Pegaptanib could bind to VEGF with higher affinity than any of its competing aptamers, inhibiting the activity of the target molecule to bind to receptors and result in overexpression. Administered through injection, clinical trials of Pegaptanib showed decrease in vision loss and was eventually approved by the FDA in 2004[12].

In light of the recent coronavirus, SARS-CoV-2 pandemic, aptamers as biological probes reveal promising biosensors for diagnosing and treating coronavirus[13]. Aptamers can detect viral

infection markers within the body including virus-stricken genes, antibodies, and proteins. With the highly sensitive nature of aptamers, they can detect and differentiate between infected or non-infected cells. Although Pegaptanib is the only aptamer- based drug approved for use by the FDA, there is hope that a similar type of drug could be designed to treat COVID-19. Researchers have found that the Helicase, a nucleic acid enzyme that binds and remodels DNA or protein complexes, may be a potent target for development in coronavirus medicinal aptamers[13]. Helicase as a target molecule for aptamer development was previously examined by Shum and colleagues in 2008. All aptamers selected from SELEX selection stimulated ATPase activate of the coronavirus helicase. Non-G-quadruplex aptamers with low Km values showed inhibition of helicase activities and were modified to increase stability invitro. The selected aptamers are undergoing further development to potentially inhibit SARS-CoV replication[14].

Biochemistry

The specific-binding nature of aptamers have allowed for developments in research focusing on chemical probing. Synthetically synthesized chemical probes allow researchers to monitor site-specific interactions of RNA aptamers. Chemical probes can be used to monitor aptamer interactions though imaging of live cellular RNAs such as chemical modifications, splicing, degradation, or translocation. Probes are especially useful in real-time analysis of optimal drug doses for patients, glucose activity in diabetic patients, or antibiotic effectiveness. For example, Ferguson and colleagues utilized a microfluidic electrochemical detector (MEDIC) in vivo while exchanging probes throughout the process to monitor a chemotherapeutic drug and an antibiotic in live rats as well as human blood[15]. MEDIC was found to achieve quantitative measurements of molecules circulating in blood in living animals. Researchers first attached a

chip to the patient's blood stream to measure drug concentration and followed this step by connecting an aptamer probe to a gold electrode. Binding of the target by the aptamer in the electrode introduced a conformational change in the aptamer-probe complex that increased the rate of electron transfer between two electrodes. Measurements were taken of current after the 3D structure change and reported as an electrochemical signal[15].

Aptamers can also be monitored by use of photoacoustic (PA) probes based on DNA aptamers[16]. The PA probe designed by Jingjing Zhang's team combined molecular recognition technology and a PA signal or shift in an absorbance peak upon target-binding to monitor aptamers. Thrombin, a molecule that plays a role in coagulation, was chosen as the target: upon aptamer binding to the target, a duplex structure was formed. Further investigation was carried out using three different control proteins as a target for the DNA probe but concluded that the original probe retained its binding specificity to thrombin even in complex environments. The aptamer based PA probe showed significant change in signals toward thrombin allowing for the first activatable PA probe to be used in molecular imaging in living animals[16].

As demonstrated above, there are a variety of ways to monitor aptamer activity through chemical probes, but the most common method involves fluorescence-based aptamer probes. Aptamer biosensors can be divided into two categories according to Wang: those that use fluorescence labels and those that employ label-free methods[17]. Analysis of aptamer activity through fluorescent probes is possible because the structural changes of aptamers upon target biding can affect the fluorescence of a dye or change the energy transfer between two dyes before and after binding. The signal change in fluorescence reflects the binding process of aptamers which allows for quantitative measurement of a target molecules concentration.

Target molecules that respond to an aptamer and reveal a change in fluorescence include proteins, small molecules, and metal ions. Aptamer based biosensors in modulation with fluorescent probes and dyes is a promising area of research in the field of disease diagnosis.

Although fluorescent RNA aptamers have been used to monitor an abundance of cellular RNA species, there are limitations in availability of dye and low brightness[8]. In 2019 a group of researchers discovered Peppers, a promising fluorescent RNA aptamer. Probe synthesis achieved (4-((2-hydroxyethyl)(methyl)amino)-benzylidene)-cyanophenyl-acetonitrile (HBC), a green fluorescent protein (GFP) fluorophore like dye. This aromatic acetonitrile is an electron acceptor that maintains rigidity and molecular structure upon binding with Peppers. Along with that, HBC was found to be non-fluorescent in solution, but fluoresces upon binding to Peppers aptamer[8] (***Figure 4***). This was useful in real time tracking of RNA activity. Specifically, live cells expressing Pepper exhibited green fluorescence when incubated with HBC. High binding affinity and thermal stability of the HBC probe are advantageous compared to previously developed fluorescent probes. It was found that the Pepper-HBC complex was not affected when fused to the ends of naturally occurring RNAs or with a terminal stem loop. In particular, Pepper was able to detect RNA polymerase and transcription of mRNAs while showing significant fluorescence in the endoplasmic reticulum and integral proteins of the plasma membrane[8]. HBC-like fluorophores showed promising results to researchers leading Chen's team to identify 7 other molecules that fluoresced from 485nm to 620nm. Chen's team found that translation of mRNA into protein was governed by normal

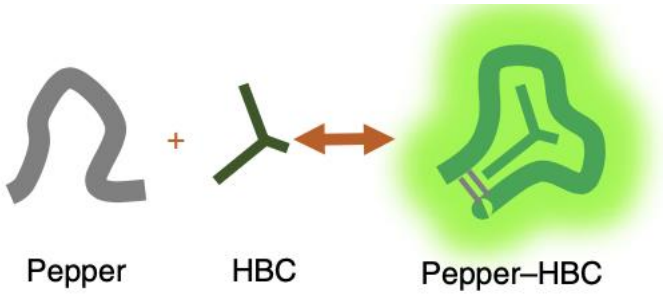


Figure 4*: Pepper-HBC Complex (Chen et al., 2019)*

enzyme kinetics but with marked heterogeneity. Along with that, Peppers can be used to image loci with CRISPR display for protein tracking.

Another fluorescent-based aptamer that monitors RNA transcription in cells is Corn. Corn can bind and induce fluorescence of 3,5-difluoro-4-hydroxybenzylidene-imidaxolinone-2-oxime (DFHO), a fluorophore that acts like red fluorescent protein (RFP)[18]. Corn was developed to quantify transcription of RNA by RNA polymerase 3 in cells. The Corn-DFHO structure was found to be a quasimetric homodimer that captures DFHO at a promoter-interface. Researchers were able to quantify RNA polymerase 3 trajectories through the photostable RNA- fluorophore complex signals that revealed mTOR inhibition, a protein kinase.

Chemistry

Retrosynthesis:

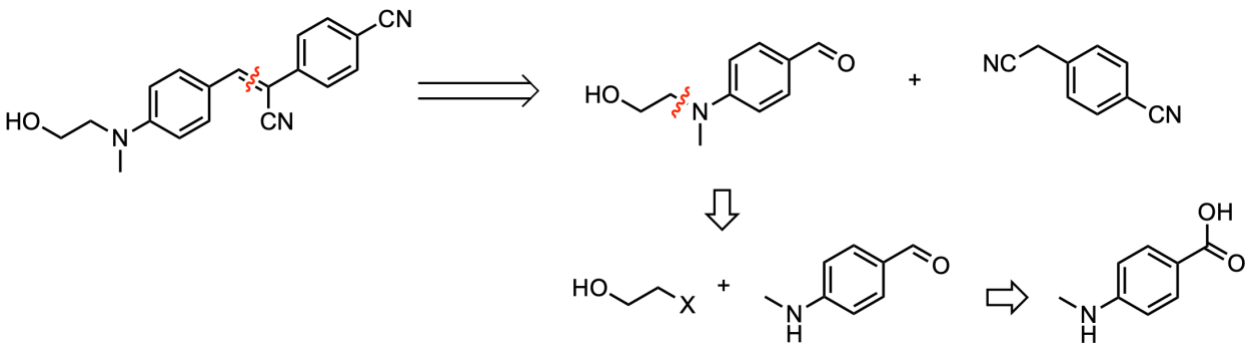


***Figure 5**: Retrosynthesis Analysis of (4-((2-hydroxyethyl)(methyl)amino)-benzylidene) cyanophenyl-acetonitrile*

Considering the ambiguity of the target molecule, (4-((2-hydroxyethyl)(methyl)amino)-benzylidene)-cyanophenyl-acetonitrile, we envisioned this multiple step retrosynthesis pathway to achieve the probe of interest (***Figure 5***). In the first step of retrosynthesis, the target molecule can be disconnected at the location of the double bond, revealing a cyano-compound and an aldehyde as potential starting material. The separation of the double bond reveals that the two starting material products can be used together in the Knoevenagel Condensation reaction. Since the cyano-compound 4-(cyanomethyl)benzonitrile can be obtained commercially, no further retrosynthesis was necessary. However, the aldehyde compound, 4-((2hydroxyethyl)(methyl)amino)benzaldehyde must be synthesized in the lab. Retrosynthesis analysis of the aldehyde revealed starting material of a hydroxyalkyl group and 4-(methylamino)benzaldehyde. These two compounds can be used to form the non-commercially available aldehyde by an N-alkylation reaction. The aldehyde can further be broken down into starting material 4-(methylamino)benzoic acid.

Synthesis

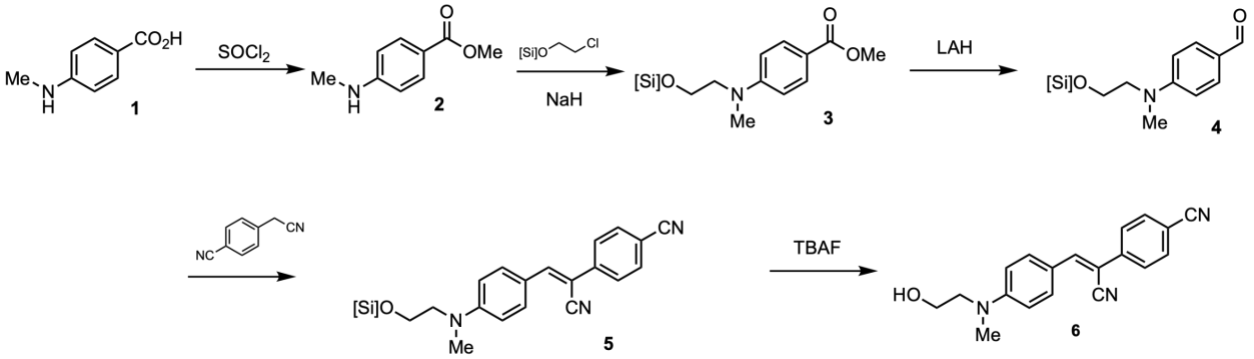


Figure 6: *Synthesis of (4-((2-hydroxyethyl)(methyl)amino)-benzylidene)* ***(6)***

In the first step of the synthesis pathway, 4-(methylamino)benzoic acid (1) underwent esterification by thionyl chloride to yield methyl 4-(methylamino)benzoate (2). The carboxylic acid was mixed with methanol and 1.5 equivalents of $SOCl_2$. To achieve methyl 4-((2-λ^1-silyl)oxy)ethyl)(methyl)amino)benzoate (3), N-alkylation was performed on the amino group of compound 2. Isolation of compound 3 allowed for further reduction of the ester group by 1 equivalent of lithium aluminum hydride (LAH) to get the aldehyde 4-((2-λ^1-silyl)oxy)ethyl)(methyl)amino)benzaldehyde (4). Because this aldehyde is not commercially available, it was necessary to synthesize compound 4 in order to perform the Knoevenagel Condensation reaction (KC). The conditions of the KC reaction should proceed as follows: reaction workup at 50°C for 48 hours, dropwise addition of strong acid followed by filtration and recrystallization from acetic acid. The catalytic base intended for use in this experiment is 1,8-Diazabicyclo(5.4.0)undec-7-ene (DBU) because it allows for the thermodynamically unfavored KC reaction to take place rather than the fast reaction. The last proposed step of the synthesis pathway involves deprotection of the alcohol to generate the target molecule (HBC).

Supplementary Information

General Experimental Procedures:

Reagents were handled in a fume hood. Column chromatography was performed with silica gel from SiliCycle (40-63 µm) with a column mixed as a slurry with the eluent and was packed, rinsed, and run under air pressure. Analytical thin-layer chromatography (TLC) was performed on precoated glass silica gel plates (by EMD Chemicals Inc.) with F-254 indicator with visualization by short wave (254 nm) ultraviolet light. Distillations were performed using a 3 cm short-path column under reduced pressure.

Instrumentation:

1 H and 13C NMR spectra were recorded on Varian Unity 500 MHz (125 MHz for 13C) spectrometer. Spectra were referenced to the residual solvent peak of CDCl3 unless otherwise noted. Chemical shifts were reported in parts per million and multiplicities are as indicated: s (singlet,) d (doublet,) t (triplet,) q (quartet,) p (pentet,) m (multiplet,) and br (broad). Coupling constants, J, are reported in Hertz and integration is provided, along with assignments, as indicated. Gas Chromatography (GC) was performed on a Shimadzu GC-2010 Plus gas chromatograph with SHRXI–MS- 15m x 0.25 mm x 0.25 µm column with nitrogen carrier gas and a flame ionization detector (FID). The glove box, MBraun LABmaster sp, was maintained under nitrogen atmosphere.

Materials:

Solvents used for extraction and column chromatography were reagent grade and used as received. Reaction solvents tetrahydrofuran (Fisher, unstabilized HPLC ACS grade), toluene (Fisher, optima ACS grade), 1,4-dioxane (Fisher, certified ACS), acetonitrile (Fisher, HPLC grade),

and hexanes (Fisher, ACS HPLC grade) were dried on a Pure Process Technology Glass Contour Solvent Purification System using activated Stainless-Steel columns while following manufacturer's recommendations for solvent preparation and dispensation unless otherwise noted. Unless otherwise shown, all noncommercial substrates were prepared according to the known literature procedures.

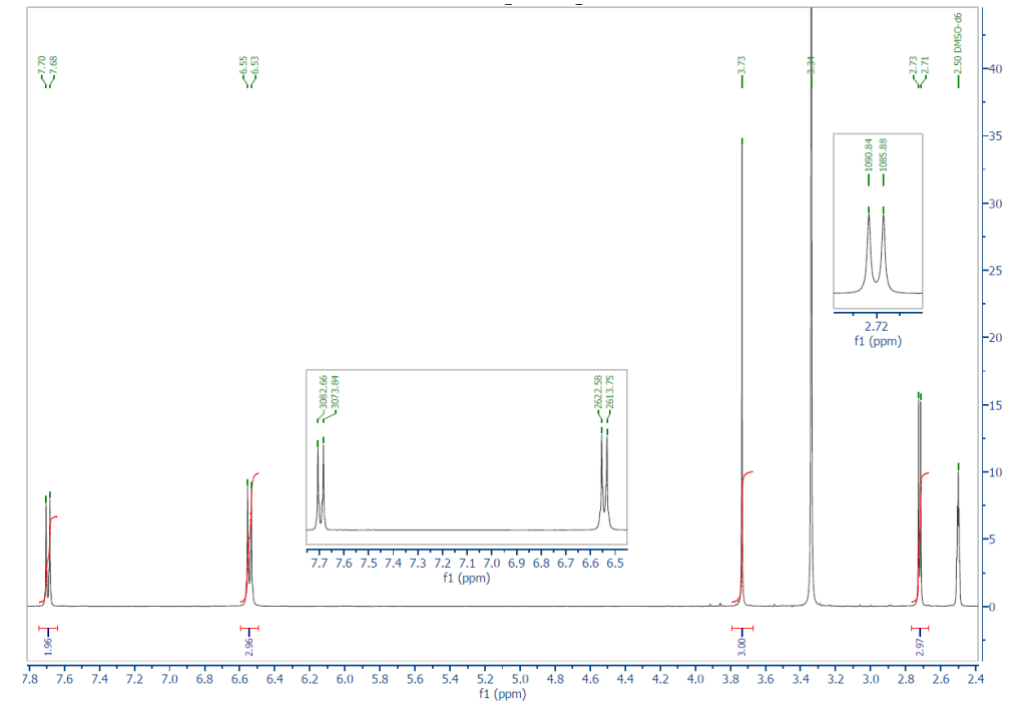
Experimental Procedures

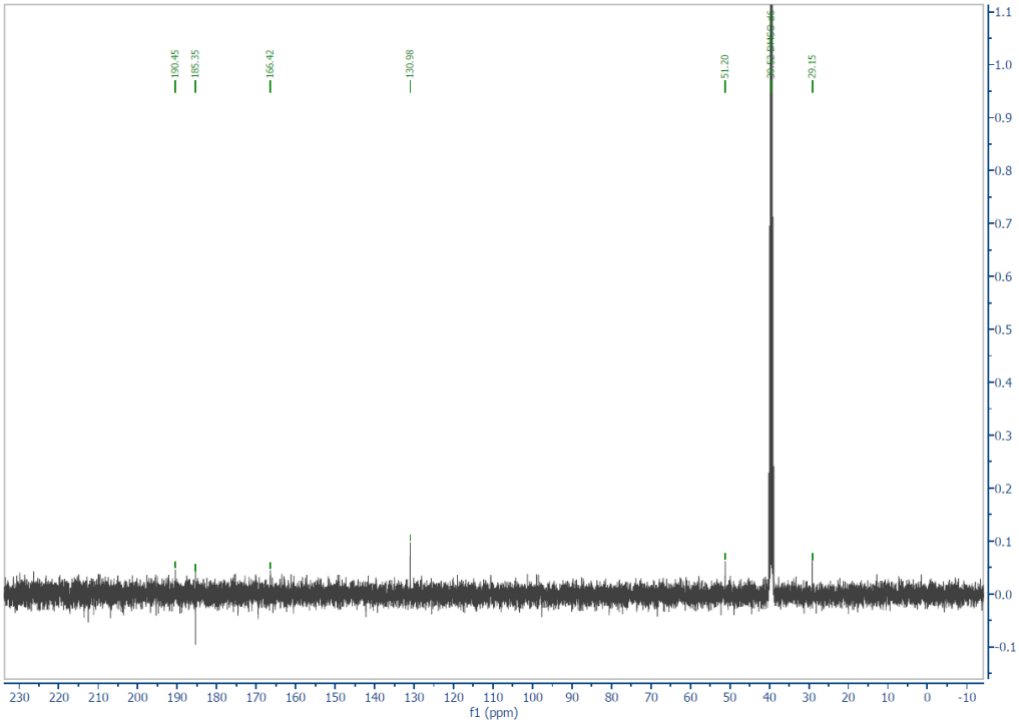
*Synthesis of methyl-4-(methylamino)benzoate **(2):***

To a solution of 4-(methylamino)benzoic acid (0.500 g, 1 Eq, 3.31 mmol) in methanol (20mL) was added thionyl chloride (590 mg, 362 µL, 1.5 Eq, 4.96 mmol) dropwise at 0°C. The reaction mixture was warmed to room temperature and stirred at 65°C for 3 hours. The reaction was allowed to cool to room temperature and was quenched with $NaHCO_3$ in an ice bath at 0°C and extracted with ethyl acetate (3 x 200mL). The organic layers were dried over Na_2SO_4 and concentrated to give methyl-4-(methylamino)benzoate **(Figure 6 (2)**). Product mass was determined to be 0.4145g (76% yield) as a yellow solid, which was used to next step without further purification.

Spectra

*methyl-4-(methylamino)benzoate **(2):***





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